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14. ABSTRACT Homeobox transcription factor Six1 has been associated with tumorigenesis and metastasis in a number of organ systems and has been implicated in proliferation, survival, and migration during normal development. Our research is aimed at utilizing mouse models to understand in the role of Six1 in the onset and progression of breast cancer. Most significantly, we have determined that Six1 is sufficient to induce tumor formation in the mammary glands of mice genetically engineered to inducibly overexpress the gene. The latency for tumor formation is between 12-24 months and the tumors that arise in these animals are very aggressive and have morphological features of an epithelial to mesenchymal transition (EMT), a phenomenon that has recently been suggested to contribute to metastasis, and stem cell origin. Molecular analysis of these tumors has revealed activation of the Wnt signaling pathway, a pathway implicated in maintaining EMT and a stem cell fate that may contribute to tumorigenesis. Additionally, we have discovered that our inducible mouse model allows for leaky transcription of Six1 in the uninduced state. Interestingly, animals from this group acquire tumors at an increased frequency compared to those animals that are induced to express Six1, suggesting that even low levels of Six1 are capable, and may even be more efficient at initiating tumorigenesis compared to higher Six1 levels. These compelling results in combination with similar findings in our experiments involving Six1 in combination with PyMT have led to more studies to dissect the role of Six1 levels and its cofactors in breast cancer initiation and progression.					
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INTRODUCTION:

Homeobox transcription factor Six1 is a critical mediator of embryonic development, where it stimulates proliferation and survival of progenitor populations as well as migration of cells (1, 2). Interestingly, Six1 expression is often found to be reinitiated in tumors where evidence suggests it stimulates proliferation and survival, and where we have most recently shown that it induces an epithelial to mesenchymal transition (EMT) in tumor cells. Together, these properties of Six1 may result in tumor progression and metastatic disease in tumors that overexpress the gene. Overexpression of Six1 is observed in numerous cancers, including breast (3-5), ovarian (6), cervical (7), and hepatocellular carcinomas (8), as well as rhabdomyosarcomas (9-11) and Wilms' tumors (12). Our research is aimed at utilizing mouse models to understand its role in the onset and progression of breast cancer. We are currently using an inducible mouse model to overexpress Six1 in differentiated cells of the mammary gland to determine if Six1 is sufficient for inducing tumor formation. Additionally, we are using a retroviral transplant model to overexpress Six1 in mammary progenitor cells to see if the gene is able to drive tumor formation differently if present in a less differentiated cell type.

BODY:

Aim 1: Determine the role of Six1 overexpression in mammary gland tumorigenesis. (Months 1-24)

(A) Evaluate the consequence of chronic Six1 overexpression in mammary gland epithelial cells using a tetracycline inducible mouse model (months 1-18).

We discovered that there is leak in our inducible system, such that we are getting low levels of Six1 expression in mammary glands taken from uninduced animals (Figure 1A). Transgene levels were significantly higher in the induced cohort as compared to the uninduced cohort, and importantly, relative *Six1* expression differences were much greater between the uninduced and induced TOSix animals than between the 4922 and 6239 transgenic lines (Figure 1A). Thus, we were able to examine dose-dependent effects of Six1 by comparing the control group (MTB +dox, expressing “no Six1”) with two different experimental groups: animals that express “low Six1” (uninduced animals from 6239 and 4922 TOSix lines); animals that express “high Six1” (dox-induced animals from 6239 and 4922 TOSix lines). Although *Six1* mRNA expression could be detected by the sensitive method of qPCR in both young and old uninduced TOSix animals, nuclear Six1 protein expression could only be detected in older, uninduced TOSix animals (Figure 1B), suggesting an age-dependent increase in Six1 protein levels over time.

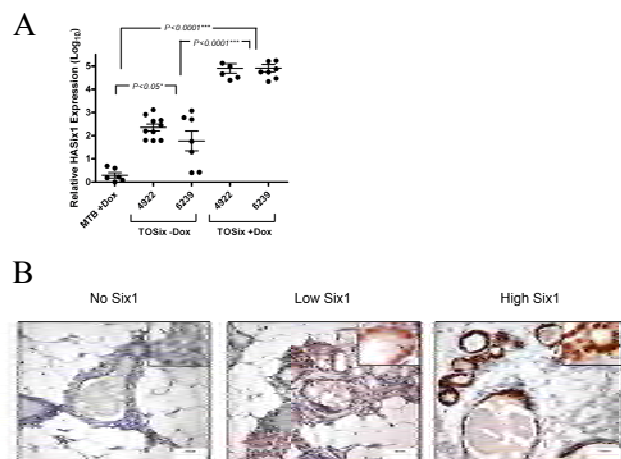


Figure 1. Characterization of the inducible, mammary-specific Six1 transgenic mouse model. (A) Real-time quantitative PCR (qPCR), using transgene specific primers and probe, reveals that *HASix1* is not expressed in the MTB control dox treated animals, but is expressed at low levels in the uninduced (-dox) mammary glands, and at high levels in the induced (+dox) mammary glands. Differences in expression between -dox and +dox mammary glands are much greater than differences between transgenic lines (4922 and 6239). Values were transformed using $\log_{10}(\text{value}+1)$ equation and plotted using a linear axis. Analysis was performed on multiparous animals and p-values were determined using two-tailed paired student's *t*-Test. (B) Immunohistochemistry using Six1 antibody reveals no Six1 protein in the control MTB+dox mammary glands (No Six1), low levels of protein in the TOSix-dox mammary glands (Low Six1), and higher levels of Six1 protein in the TOSix+dox mammary glands (High Six1) (Scale bar = 100um). Clear nuclear staining is shown in higher magnification insets (arrow).

Long term induction of Six1 expression (TOSIX bitransgenic animals overexpress the gene upon treatment with doxycycline) results in mammary tumor formation in a subset of animals after approximately 12-21 months. Interestingly, tumors actually arise more frequently in these animals (40%) compared to those induced to express Six1 (20%), suggesting that there may be some dose-dependency to Six1's ability to initiate tumorigenesis (Figure 2A). These tumors have been characterized by our collaborating pathologist, who determined that they are high grade adenocarcinomas with histologically diverse features (Figure 2B).

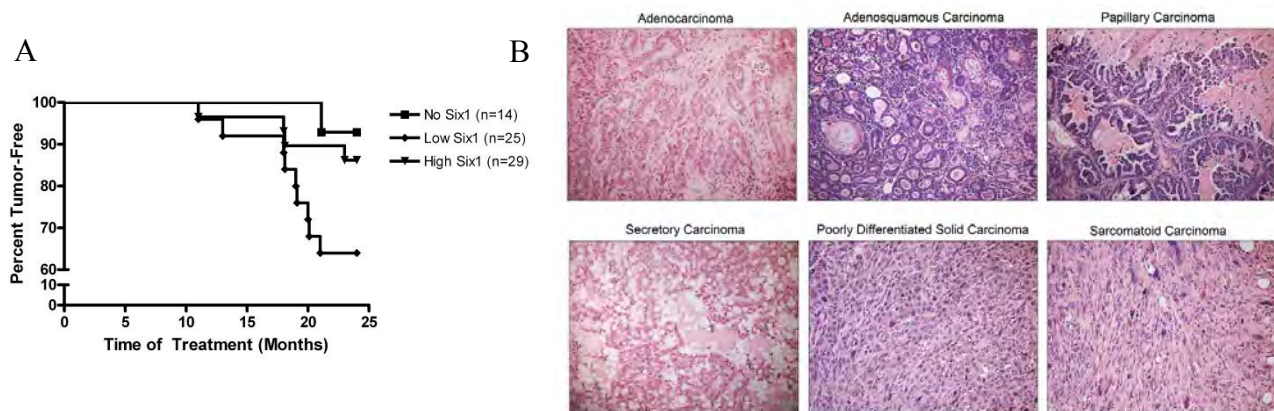


Figure 2. Mammary tumors arise in Six1-expressing animals in a dose-dependent manner and manifest histologically diverse phenotypes. (A) Kaplan-Meier analysis of percent tumor-free animals reveals that both low Six1 and high Six1 animals develop tumors, but tumor frequency is higher in low Six1-expressing animals. (B) Shown are representative images of H&E stained tumor sections demonstrating various histological patterns of tumors observed in TOSix animals.

These include features of EMT, including sarcomatoid regions that are characterized by loss of E-cadherin expression (a marker of epithelial cells), gain of smooth muscle actin (a marker of mesenchymal cells), and retention of cytokeratin 18 (suggesting epithelial origin) (Figure 3). We detected lung masses in some of the animals and originally believed that they were metastases. However, immunohistochemical staining revealed that the tumors are of lung origin, suggesting that these animals acquired primary lung tumors, a common occurrence in aged mice. Analysis of molecular markers in the tumors revealed the expression of nuclear β -catenin, as well as its transcriptional target cyclin D1, in approximately 70% of Six1-driven tumors, suggesting activation of the Wnt signaling pathway (Figure 4A). qPCR analysis was performed to detect other transcriptional targets of the Wnt signaling pathway, including *Tcf7*, *Axin2*, *c-Myc*, and *cyclin D1*. Interestingly, these targets were upregulated in Six1-driven tumors, but not in the Six1-overexpressing mammary glands (Figure 4B). These results suggest that activation of the Wnt signaling pathway either occurs as a preferred cooperating oncogenic mutation in Six1-mediated tumorigenesis, or that Six1 is able to initiate Wnt signaling specifically in the tumorigenic context. Interestingly, *ezrin*, a previously reported target of Six1 in rhabdomyosarcomas (11) is also specifically expressed in Six1-driven tumors, suggesting that Six1 can activate this target specifically in the tumorigenic context in the mammary gland (Figure 4C).

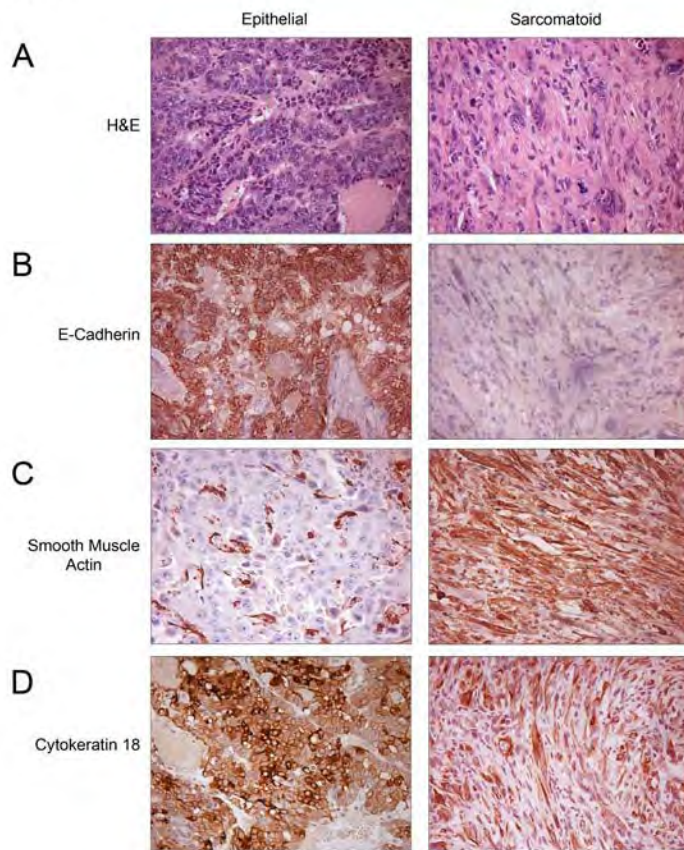


Figure 3. A subset of Six1 tumors show a complete EMT. (A) H&E stained sections of regions of tumor showing epithelial and sarcomatoid (spindle-cell) morphology. (B) Immunohistochemistry (IHC) with E-cadherin antibody shows strong cell-surface staining in the epithelial regions, and complete absence of staining in the sarcomatoid regions. (C) IHC to detect the EMT marker smooth muscle actin (SMA) shows a gain of SMA expression in the sarcomatoid regions. (D) IHC performed using an antibody against the luminal epithelial marker cytokeratin 18 (CK18). Sarcomatoid tumors retain cytokeratin expression, supporting an epithelial origin.

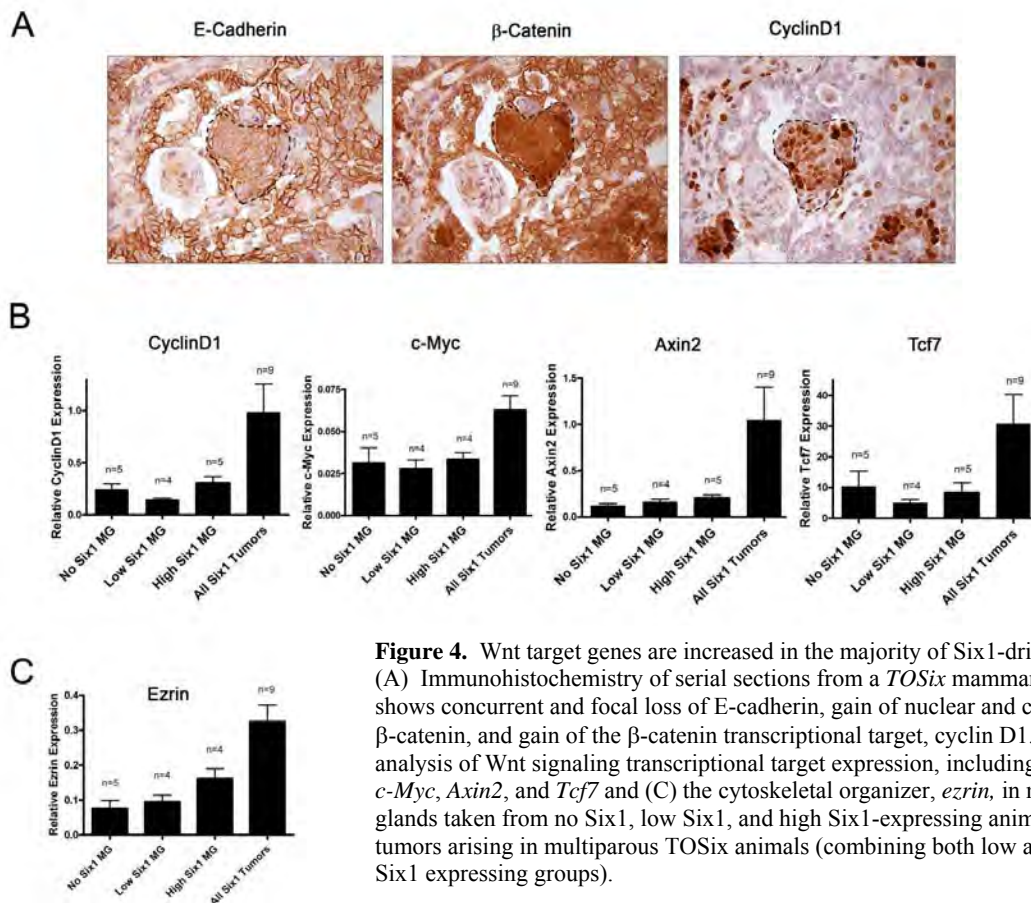


Figure 4. Wnt target genes are increased in the majority of Six1-driven tumors. (A) Immunohistochemistry of serial sections from a TOSix mammary tumor shows concurrent and focal loss of E-cadherin, gain of nuclear and cytoplasmic β-catenin, and gain of the β-catenin transcriptional target, cyclin D1. (B) qPCR analysis of Wnt signaling transcriptional target expression, including *cyclin D1*, *c-Myc*, *Axin2*, and *Tcf7* and (C) the cytoskeletal organizer, *ezrin*, in mammary glands taken from no Six1, low Six1, and high Six1-expressing animals versus tumors arising in multiparous TOSix animals (combining both low and high-Six1 expressing groups).

Six1 is known to stimulate proliferation in progenitor populations during normal development (2, 4, 6, 13-17), and recent evidence suggests that the closely related family member Six2 may contribute to the self-renewing potential of kidney stem cells (18). Six1-overexpressing tumors display features of Wnt signaling activation, a pathway which has been strongly implicated in mammary stem cell maintenance (19). Additionally, Six1-driven tumors undergo EMT, a process strongly associated with the gain of stem cell characteristics (20). Therefore, we sought to determine whether Six1 could promote a stem/progenitor cell phenotype in mammary epithelial cells and whether Six1-driven tumors would exhibit stem/progenitor cell characteristics.

To determine whether Six1 overexpression alters the percentage of mammary stem cells, primary mammary epithelial cells were isolated from low Six1-expressing females from both the 6239 and 4922 transgenic lines and no Six1 control females aged approximately 1.5 years. We chose to analyze the sucrose-treated cohort, as these animals presented the highest frequency of tumor formation. We also analyzed primary mammary epithelial cells harvested from 8 week old FVB and 1 year old FVB wild type mice to control for age-related differences in stem cell populations. Epithelial cells were analyzed by flow cytometry using the stem cell markers CD24 and CD29. Interestingly, aged FVB control animals, as well as aged no Six1 animals, displayed a very small CD24⁺CD29⁺ population (16.7% and 16.1%, respectively) (Figure 5A). However, aged low Six1-expressing animals from both transgenic lines displayed an increased CD24⁺CD29⁺ population compared to no Six1 controls (32.0% and 28.4% vs. 16.1%). Interestingly, the percentage of stem cells observed in the aged, low Six1 mammary glands was similar to the 8 week old FVB control animals (30.5%) (Figure 5B).

To further determine whether the increased mammary stem cell population in Six1 overexpressing animals is functional, mammosphere assays were performed using epithelial cells isolated from the above groups. The secondary mammosphere assay, which measures capacity for self-renewal (21-23), revealed that Six1-overexpressing mammary glands contain an increased number of functional mammary stem cells. Similar to the results from flow cytometry, mammary cells isolated from aged no Six1 and FVB control animals formed very few mammospheres. However, mammary epithelial cells isolated from aged low Six1-expressing animals from both transgenic lines formed a similar number of mammospheres as 8 week old FVB control animals (Figure 5B). These results demonstrate that mammary glands overexpressing Six1 are enriched for stem cells, suggesting that Six1 may play a role in the establishment or maintenance of mammary stem cell populations.

To determine if Six1-driven tumors also manifest a stem/progenitor cell phenotype, we examined the TOSix tumors for expression of the luminal epithelial cell marker cytokeratin 18 (CK18), the myoepithelial cell marker cytokeratin 5 (CK5), and the mammary progenitor cell marker CK6 (24). Strikingly, Six1-driven tumors exhibited areas of mixed cytokeratin expression, including CK18, 5, and 6 (Figure 6A). Additionally, 9/12 (75%) TOSix tumors expressed the stem cell marker Sca-1 within the tumor at the following frequency: less than 5% of the tumor (4/12), between 5-15% of the tumor (3/12), or greater than 15% of the tumor (2/12) (Figure 6B). These data, in conjunction with the observation that individual tumors driven by Six1 frequently show multiple histologic patterns and activation of Wnt-signaling, suggest that Six1-induced tumors arise from a progenitor/stem-like cell population.

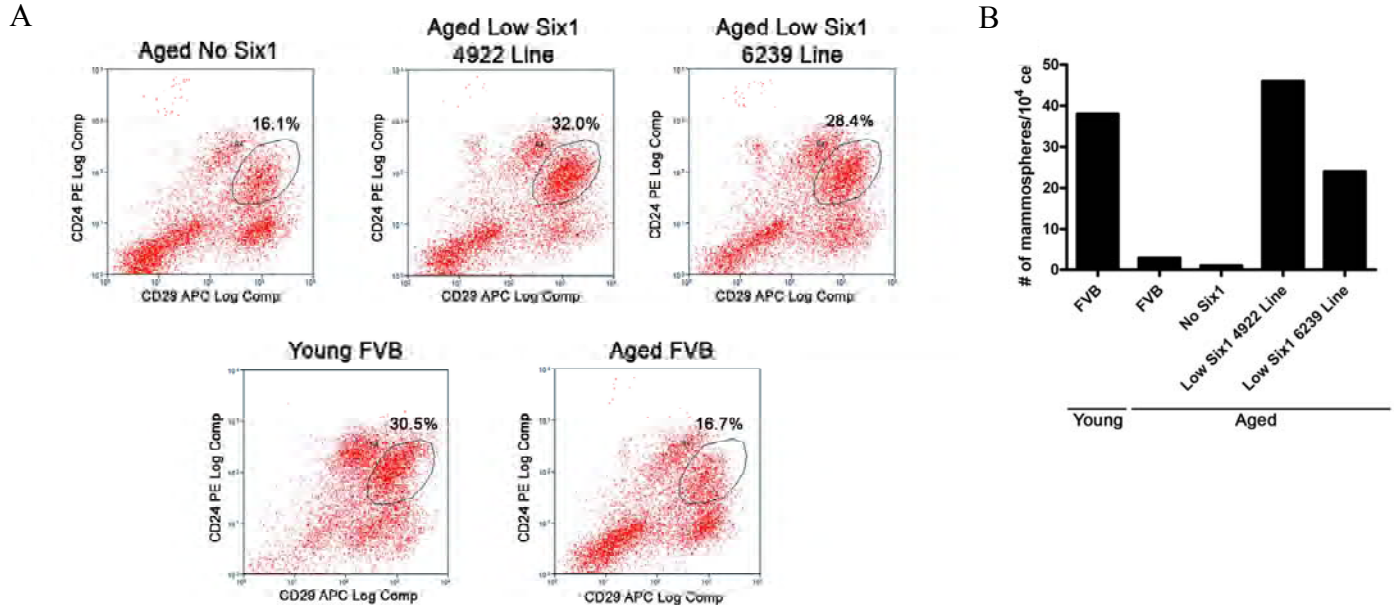


Figure 5. Six1-overexpressing mammary glands exhibit stem/progenitor cell characteristics. (A) Flow cytometry analysis of mammary epithelial cells harvested and pooled from 3 animals per group: 1.5 year old low Six1-expressing animals from 4922 and 6239 lines (aged); 1.5 year old no Six1 animals (aged); 1 year old FVB wild type animals (aged); and 8 week old FVB wild type animals (young). Low Six1-expressing animals are enriched for mammary stem cells compared to aged FVB wild type and no Six1 control animals, as measured by the CD24⁺CD29⁺ population. Low Six1-expressing animals have a similar percentage of mammary stem cells as 8 week old FVB wild type mice. (C) Secondary mammosphere assays were performed using epithelial cells isolated from the groups identified in (B). Mammosphere numbers are increased in low Six1-expressing animals compared to no Six1 and aged FVB controls and are similar to young FVB controls.

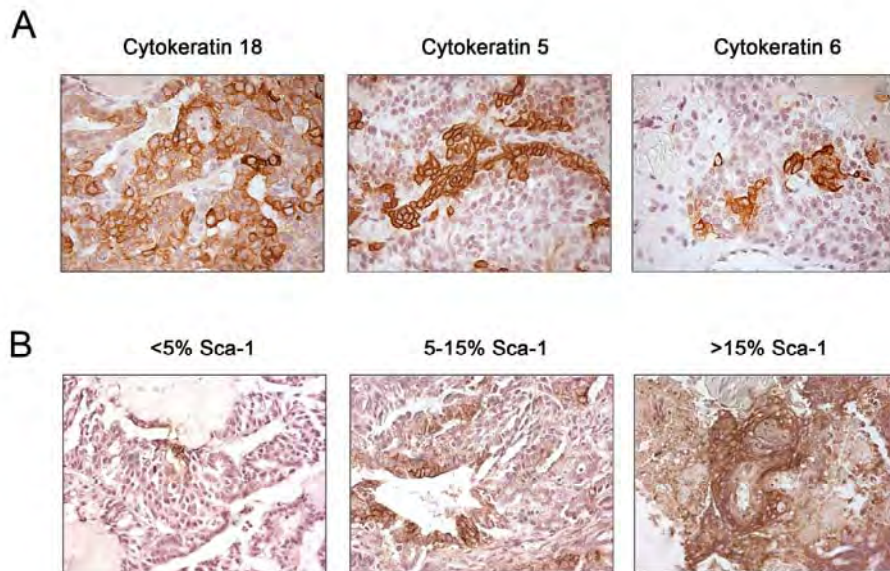


Figure 6. Six1-driven tumors display features of stem/progenitor cell origin. (A) Immunohistochemistry of sections from a TOSix mammary tumor with activated Wnt signaling displays mixed expression of cytokeratins marking different cell types, including cytokeratin 18 (CK18) which is expressed on luminal epithelial cells, cytokeratin 5 (CK5), which is expressed on myoepithelial cells, and cytokeratin 6 (CK6), which is expressed on the surface of mammary progenitor cells. (B) Immunohistochemistry of sections from TOSix mammary tumors displaying expression pattern of Sca-1 staining (expressed in less than 5% of the tumor, between 5-10% of the tumor, and greater than 15% of the tumor).

In addition to the tumor phenotype, we observe a hyperplastic phenotype in the mammary glands of animals induced to overexpress Six1 (previously described in 2007 report).

(B) Determine the effect of retroviral-mediated Six1 overexpression in mammary gland progenitor cells (months 1-18).

In the last year, we have been using a retroviral transduction method to overexpress Six1 in the mammary gland in combination with the polyoma middle T antigen (PyMT), a potent oncogene, to determine if Six1's expression is able to shorten tumor latency or increase PyMT-mediated lung metastasis. In order to do this, we have harvested primary mammary epithelial cells from wildtype animals and then retrovirally transduced them with Six1 and PyMT tagged with GFP (controls including Six1 alone or PyMT alone are included). These cells are then transplanted into the cleared fat pad of three week old recipient wildtype mice. Progenitor cells within the population will give rise to a mammary ductal network that will express Six1 along with PyMT.

PyMT is known to give rise to tumors with a very short latency in this model (approximately 3 months following transplant), and consistently leads to lung metastasis when tumors grow to a large size, or when the primary tumor is removed (25). In our original study, we obtained an infection efficiency of approximately 15%. In this study Six1 did not affect primary tumor latency or growth, but led to a significant increase in the frequency of lung metastasis, as well as an increase in metastatic burden. In a second experiment to confirm these results, we obtained a much higher infection efficiency of approximately 40%. Interestingly, in this experiment, the expression of Six1 prevented tumor formation and appears to have initiated mammary differentiation (Table 1 and Figure 7).

Table 1

Approximate Infection Efficiency	Phenotype	Cells Transplanted	Number
15%	Metastasis	PyMT	1/5
		PyMT + Six1	6/7
40%	Tumor	PyMT	9/10
		PyMT+Six1	2/10

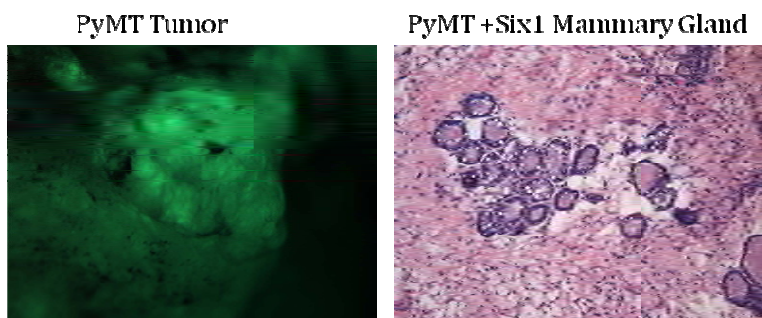


Figure 7. PyMT with Six1 at high infection efficiency leads to mammary differentiation. Fluorescent image of tumor from animal injected with mammary cells transduced with PyMT alone (3 months after injection) (left). H&E of mammary gland from animal injected with mammary cells transduced with Six1 and PyMT (4 months after injection) showing differentiation (right).

These results suggest that perhaps, as already mentioned above, Six1 levels may be critical in mediating its effects on tumorigenesis. Other cases for oncogene dose-dependency have been cited in the literature involving Ras, as well as Oct3/4 (26, 27). We believe that carefully dissecting the importance of Six1 expression levels is critical for understanding its role in human breast cancer and for developing therapies for appropriately targeting its function. At this time, we are planning experiments to titrate Six1 expression levels and determine whether there is an optimal level of Six1 expression for its pro-tumorigenic role, and that, perhaps at higher levels, it is actually anti-tumorigenic. In our experience, performing these types of titration experiments in animals is difficult. Therefore, we will pursue these experiments using cell culture techniques before moving on to animal studies. As these experiments will be quite involved, they are outside the scope of this grant.

Our studies involving combining Six1 with a dominant-negative form of p53 have not resulted in any tumors, suggesting that Six1 is not collaborating with p53 to lead to mammary tumorigenesis, at least in this model.

(C) Determine if mammary gland tumorigenesis (if observed) or additional phenotypes are dependent upon Six1 for maintenance using the inducible and retroviral overexpression models outlined in aims 1a and 1b (months 18-24).

At this time, due to the discovery that this inducible system allows for leaky expression of Six1 that is sufficient for inducing a phenotype, we do not believe that the completion of this aim will give us interpretable data.

Aim 2: Examine the dependency of Six1 on cyclin A1 for mammary gland proliferation and tumorigenesis. (Months 18-36)

As the tumor latency with Six1 overexpression is long, we have not begun the experiments associated with this aim where we will cross TOSIX1 mice with Cyclin A1 knockout mice to determine whether cyclin A1 is required for Six1-mediated tumorigenesis. We are generating MMTV-Six1 transgenic mice so that this experiment can be done more easily, without the necessity of bitransgenic lines and doxycycline treatment. Developing the MMTV-Six1 mouse model is beyond the scope of this grant.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified that long term induction of Six1 expression in an inducible mouse model leads to hyperplasia as well as aggressive tumor formation.
- Characterized the tumors to identify a Six1-induced epithelial-to-mesenchymal transition, as well as the presence of active Wnt signaling using molecular markers.
- Identified that the tumors also show markers of having arisen from more progenitor like cells, including keratin 6 and Sca-1 expression, and expression of mixed markers of myoepithelial and luminal epithelial lineages (keratin 5 and 18, respectively).
- Demonstrated that Six1 promotes a stem/progenitor cell phenotype, both in a normal and tumorigenic context.
- Identified leaky expression in the inducible model that results in low levels of Six1 expression in the uninduced mammary glands, leading to increased tumor frequency, suggesting a Six1 dose-dependency.

- Noted that Six1 does not affect tumor latency or growth when combined with the PyMT oncogene.
- Discovered that at low infection efficiency, Six1 is able to enhance PyMT-mediated lung metastasis and that at high efficiency, Six1 is able to prevent PyMT-mediated tumorigenesis and may induce differentiation.
- Determined that Six1 does not collaborate with p53DN to initiate tumorigenesis in this model.

REPORTABLE OUTCOMES:

Research presented in the form of a short talk (invited based on abstract submission) and poster at the Gordon Conference for Mammary Gland Biology in Newport, RI, June 2007, as well as at the Era of Hope Meeting in Baltimore, MD, June 2008. Research was also presented in the form of a poster at the Keystone Meeting for Signaling Pathways in Development and Cancer in Steamboat Springs, CO, March 2008 for which my abstract was selected for a Keystone Symposia travel award scholarship. Research was also presented at the Gordon Conference for Mammary Gland Biology in Barga, Italy, June 2008 in the form of a poster.

The following manuscript resulted from this work:

McCoy, EL, Iwanaga, R, Jedlicka, P, Abbey N-S, Chodosh, LA, Heichman, K, Welm, AL, and HL Ford. "The Six1 Homeoprotein Induces Mammary Tumors with Epithelial-Mesenchymal-Transition and Stem/Progenitor Cell Characteristics." (Manuscript under revision at *The Journal of Clinical Investigation*).

Grants that resulted from this work include a grant from the American Cancer Society entitled "The Role of Six1 in EMT and Tumor Progression". (per year direct costs/ for 4 years.

The grant runs from 5/1/07-4/30/11 and 2R01-CA095277 -06 (Ford), "*The role of Six1 in EMT and Tumor Progression*" 9/29/2007 – 9/28/2012 from NIH/NCI (in directs, all overlap removed and budget reduced to avoid overlap with ACS grant).

CONCLUSIONS:

Our data are the first to demonstrate that Six1 overexpression is sufficient to induce tumorigenesis when expressed out of context in a normal adult cell, leading to highly aggressive and invasive mammary tumors with EMT and stem cell features. We conclude that Six1 is promoting tumorigenesis by re-employing its developmental program to drive not only proliferation and survival, but also migration and invasion through EMT. Additionally, our data are the first to suggest that Six1 acts to promote a stem cell phenotype. These results are quite compelling, given the clinical data that Six1 is overexpressed in 50% of primary breast cancers and 90% of metastatic lesions (3-5). Our ultimate goal is to identify Six1 as a legitimate therapeutic target. As Six1 expression is primarily expressed only in embryogenesis, lost in the adult, and re-expressed in cancers, targeting Six1 in a clinical setting may successfully treat cancer while avoiding damage to normal adult tissues, thus limiting side-effects. The importance of understanding a possible Six1 dose-dependency is critical for our future attempts to control Six1 as a cancer therapy.

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APPENDICES AND BIBLIOGRAPHY:

Personnel Receiving Pay for Research Effort

Erica McCoy

Manuscripts

McCoy, EL, Iwanaga, R, Jedlicka, P, Abbey N-S, Chodosh, LA, Heichman, K, Welm, AL, and HL Ford. "The Six1 Homeoprotein Induces Mammary Tumors with Epithelial-Mesenchymal-Transition and Stem/Progenitor Cell Characteristics." (Manuscript under revision at *The Journal of Clinical Investigation*).

Presentation Abstracts

Poster/talk abstract presented at the Gordon Conference on Mammary Gland Biology, Newport, RI, June, 2007 (selected for short talk based on the submission of this abstract):

In Vivo Role of the Six1 Homeoprotein in Mammary Gland Tumorigenesis

E.M. McCoy¹, N. Abbey², P. Jedlicka³, L. Chodosh⁴, H.L. Ford^{1,2}, ¹Program in Molecular Biology, ²Department of Obstetrics and Gynecology, ³ Department of Pathology, University of Colorado Health Sciences Center, Denver, Colorado, USA ⁴Department of Cancer Biology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA

Human Six1 is a homeodomain-containing transcription factor that is critical for cell proliferation, survival, and epithelial to mesenchymal transition (EMT) during normal development. In addition to its developmental role, overexpression of Six1 has been detected in a number of human cancers, including breast cancer, where it is linked to both proliferation and metastasis. As many as 50% of primary breast cancers and 90% of metastatic lesions overexpress the gene, in part due to gene amplification. Six1 can transform a mammary epithelial cell line, but no work has been done to show the effects of Six1 overexpression *in vivo*. We have established an inducible, mammary-specific Six1 overexpression model by crossing MMTV-rtTA mice to TetO-Six1 mice, and are using this model to test whether Six1 overexpression leads to mammary tumors, as well as to dissect the molecular mechanism by which Six1 influences tumorigenesis *in vivo*. In mice induced to constitutively overexpress Six1 in the mammary gland, marked hyperproliferation and abnormal alveologenesis is observed. In addition, tumor formation is observed after long latency (>1 year). Tumors formed are complex, but are best characterized as invasive ductal adenocarcinomas with complex features. The tumors contain regions with papillary and secretory differentiation, as well as high grade solid areas. Most importantly, sarcomatoid differentiation (spindle cell morphology) is observed, and E-cadherin expression is lost in high grade areas of the tumor. Thus, this transgenic model demonstrates that inappropriate expression of Six1 promotes high grade tumor formation and oncogenic EMT, suggesting that Six1 is important not only for tumor initiation, but also for tumor progression. This inducible model provides us with a system to examine whether removal of Six1 expression can reverse the phenotypes, thereby addressing whether Six1 is a viable drug target.

Importantly, Six1 is not necessary for most normal adult tissues, and thus therapies directed against Six1 may not lead to the severe side effects seen with more conventional treatments.

Poster abstract presented at the Keystone Symposia on Signaling Pathways in Development and Cancer, Steamboat Springs, CO, March 2008 and the Era of Hope meeting, Baltimore, MD, June, 2008 (abstract selected for Keystone Symposia travel award scholarship and for short talk at the Era of Hope meeting):

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E.M. McCoy, Alana Welm, Karen Heichman, P. Jedlicka, L. Chodosh, H.L. Ford. Program in Molecular Biology, University of Colorado Health Sciences Center, Aurora, Colorado, USA, 80045

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Poster abstract presented at the Gordon Conference on Mammary Gland Biology, Barga, Italy, June 2008:

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